JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1712–1719 Vol. 178, No. 6
0021-9193/96/$04.00+0


# Mutations within the First LSGGQ Motif of Ste6p Cause Defects in **a**-Factor Transport and Mating in *Saccharomyces cerevisiae*

BARCLAY L. BROWNE, VERA MCCLENDON, AND DAVID M. BEDWELL*

*Department of Microbiology, The University of Alabama at Birmingham, Birmingham, Alabama 35294*

Received 30 August 1995/Accepted 10 January 1996

**Mating between the two haploid cell types (a and α) of the yeast *Saccharomyces cerevisiae* depends upon the efficient secretion and delivery of the a- and α-factor pheromones to their respective target cells. However, a quantitative correlation between the level of transported a-factor and mating efficiency has never been determined. a-Factor is transported by Ste6p, a member of the ATP-binding cassette (ABC) family of transporter proteins. In this study, several missense mutations were introduced in or near the conserved LSGGQ motif within the first nucleotide-binding domain of Ste6p. Quantitation of extracellular a-factor levels indicated that these mutations caused a broad range of a-factor transport defects, and those directly within the LSGGQ motif caused the most severe defects. Overall, we observed a strong correlation between the level of transported a-factor and the mating efficiency of these strains, consistent with the role of Ste6p as the a-factor transporter. The LSGGQ mutations did not cause either a significant alteration in the steady-state level of Ste6p or a detectable change in its subcellular localization. Thus, it appears that these mutations interfere with the ability of Ste6p to transport a-factor out of the *MAT***a** cell. The possible involvement of the LSGGQ motif in transporter function is consistent with the strong conservation of this sequence motif throughout the ABC transporter superfamily.**

The two haploid cell types of the yeast *Saccharomyces cerevisiae* (**a** and α) are able to recognize one another and fuse to form diploid (**a**/α) cells. Efficient mating depends upon the correct expression of haploid-specific gene products, including the diffusible mating pheromones **a**- and α-factor. The secretion and delivery of these mating pheromones to target cells of the opposite mating type initiate the complex cascade of cellular responses that lead to diploid formation. Since *S. cerevisiae* is a nonmotile organism, each haploid cell must locate an appropriate proximal mating partner and properly orient itself in relationship to this partner in order to fuse with it. Hartwell and coworkers have shown that the amount of either **a**- or α-factor secreted by each haploid cell type is the primary determinant of partner selection (partner discrimination) during the early, courtship phase of yeast mating (19–21). A *MAT*α cell, for example, when coincubated with two different *MAT***a** strains secreting different levels of **a**-factor, will mate most frequently with the *MAT***a** strain secreting the highest level of **a**-factor. *MAT***a** yeast cells containing deletions of the two genes encoding **a**-factor, *MFa1* and *MFa2*, are unable to produce **a**-factor and are sterile (7, 34). The addition of exogenous **a**-factor to this mating mixture restores less than 1% of wild-type diploid formation. Moreover, the addition of exogenous **a**-factor to a mating mixture containing both wild-type *MAT***a** and *MAT*α yeast cells inhibits mating to a level that is less than 1% of that observed when the two strains are coincubated without the pheromone (31, 34). These data suggest that yeast cells are unable to respond appropriately to a uniform field of pheromone and have led to the proposal that efficient yeast mating requires the establishment of a pheromone gradient from the secreting cell to the target cell in order to provide the positional cues necessary for diploid formation (19). Given the above, an accurate analysis of the dependence of yeast mating and its constituent processes upon the amount of extracellular **a**-factor will require the titration of different levels of transported **a**-factor in a native context, i.e., as exported from *MAT***a** yeast cells.

The **a**-factor pheromone is a dodecapeptide and is both carboxymethylated and farnesylated at its C-terminal cysteine residue (3, 42). It matures intracellularly and is then transported out of *MAT***a** yeast cells by the *STE6* gene product, Ste6p (28, 33, 44, 50). The assignment of Ste6p as the **a**-factor transporter stems from the observation that disruption of the *STE6* gene blocks **a**-factor transport and causes the intracellular appearance of the mature pheromone (28). In addition, *sec* mutations that block protein transport through the secretory pathway (including α-factor maturation and secretion) have no effect on **a**-factor secretion, indicating that **a**-factor transport is independent of the classical secretory pathway (22, 28, 33).

Ste6p belongs to a large protein superfamily called either the ATP-binding cassette (ABC) transporter family (17) or the traffic ATPase family (1). Representatives of this protein family are found in species ranging from *Escherichia coli* to humans and function to transport a wide variety of substrates directly across membranes in an ATP-dependent manner (1, 2, 17). ABC transporters are predicted to consist of a pair of repeated elements, each having a hydrophobic, integral membrane domain followed by a nucleotide-binding domain (NBD) (Fig. 1A). These NBDs retain a significant degree of amino acid sequence homology and contain the Walker A (GxxGxGKS/T) and Walker B ($Rx_{6-8}h_4D$) motifs, where x is any amino acid and h is a hydrophobic amino acid, common to many proteins that bind ATP and GTP (36, 41, 49). The NBDs also contain a third consensus element, the LSGGQ (or C) motif, located just N-terminal to the Walker B motif (Fig. 1B).

* Corresponding author. Mailing address: Department of Microbiology, Bevill Biomedical Research Building, Rm. 432, The University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294-2170. Phone: (205) 934-6593. Fax: (205) 975-5479. Electronic mail: david__bedwell@micro.microbio.uab.edu.

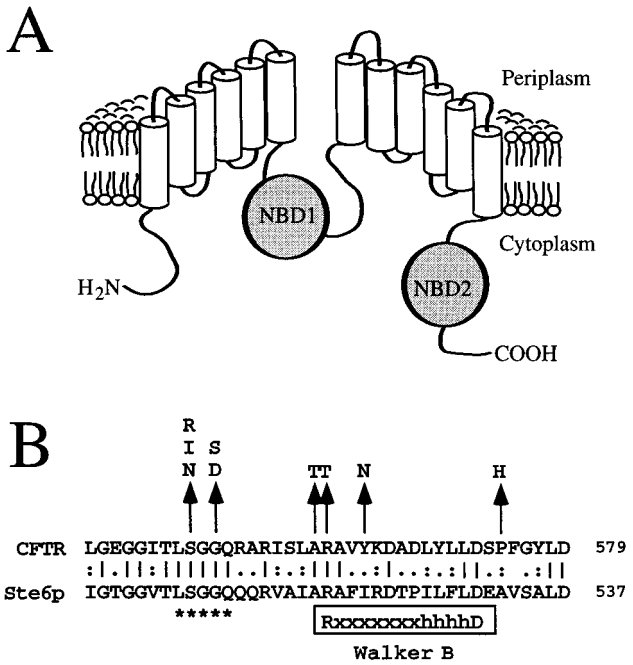


FIG. 1. (A) Predicted topological model of Ste6p in the plasma membrane. (B) Amino acid sequence alignments of the LSGGQ region of the first NBDs of CFTR and Ste6p. Maximal pairwise alignments of protein sequences were carried out with the Gap program of the Genetics Computer Group (a gap weight of 3.0 and gap length weight of 0.1 were used) (11). Numbers indicate the position of the aligned amino acid sequences in each protein. The consensus Walker B motif (41, 49) is boxed. The conserved LSGGQ sequence is underscored with asterisks. Naturally occurring CFTR missense mutations used in this study are indicated above the position where each mutation has been identified (10, 23, 47). In the Walker B motif, x represents any amino acid and h represents hydrophobic amino acids.

Several mutations which cause the disease cystic fibrosis (CF) are clustered in or near the LSGGQ sequences of another ABC transporter, the CF transmembrane conductance regulator protein (CFTR) (10, 23, 47; for a review, see reference 48). From such observations, it was proposed that the LSGGQ motif mediates a conserved, essential function among ABC transporters (1).

In this study, nine CF-associated missense mutations were introduced in or near the LSGGQ motif within the first NBD of the **a**-factor transporter Ste6p. We found a positive correlation between the level of transported **a**-factor and the efficiency of yeast mating, consistent with the role of Ste6p as the **a**-factor transporter. Under conditions in which the level of transported **a**-factor is limiting for yeast mating, small changes in the amount of transported pheromone caused large alterations in the mating efficiency. This indicates that *MAT*α cells can efficiently discriminate between small differences in the level of **a**-factor presented by *MAT***a** cells. None of these mutations caused detectable changes in either the steady-state abundance or the localization of Ste6p, suggesting that the LSGGQ motif within the first NBD of Ste6p plays a direct role in its transporter function.

## MATERIALS AND METHODS

**Strain and plasmid construction.** The Δ*ste6* yeast strains were constructed by the one-step gene replacement procedure (39) as previously described (28). Briefly, an internal 3.4-kb *Stu*I-*Sna*BI fragment was excised from plasmid pBR322-*STE6* (50) and replaced with a 1.8-kb *Bam*HI fragment containing the *HIS3* gene by blunt-end ligation. A 2.6-kb *Spe*I-*Ssp*I fragment bearing the *HIS3* gene flanked by *STE6* sequences was then gel purified and electroporated into the yeast strains indicated below. $His^+$ colonies were selected, and proper integration of the *ste6*::*HIS3* construct was confirmed by Southern analysis and the **a**-factor halo assay. The strains used in this study were YDB108 (*MAT***a** *ura3-52 Δhis3-200 trp1-Δ901 ade2-101 leu2-3,112 Δpep4*::*LEU2 Δste6*::*HIS3*), YDB141 (*MAT***a** *ura3-52 Δhis3-200 trp1-Δ901 ade2-101 Δste6*::*HIS3*), and YDB199 (*MAT***a** *ura3-52 Δhis3-200 ade2-101 act1-1 Δste6*::*HIS3*).

To introduce mutations into the *STE6* gene, a 1.2-kb *Pst*I fragment was removed from plasmid pBR322-*STE6* and subcloned into M13mp18 (51). Mutations were then introduced by site-directed mutagenesis (29) and confirmed by DNA sequence analysis (40). The 1.2-kb *Pst*I fragment was then subcloned back into the *STE6* gene under control of the *STE6* promoter in both the low-copy-number plasmid pSEYC58 and the high-copy-number plasmid pSEY8 (13), both containing the *URA3* gene as the selectable marker. The resulting low-copy vectors were pDB193 (Ste6p wild type [WT]), pDB181 (Ste6p-S507N), pDB156 (Ste6p-S507R), pDB153 (Ste6p-S507I), pDB184 (Ste6p-G509D), pDB159 (Ste6p-G509S), pDB132 (Ste6p-A517T), pDB138 (Ste6p-R518T), pDB141 (Ste6p-I521N), and pDB144 (Ste6p-A532H). For **a**-factor overproduction, a 1.6-kb *Bam*HI fragment containing the *MFa1* gene was cloned into the high-copy vector Yeplac112, which contains the *TRP1* gene as a selectable marker (14), yielding pDB360. Transformations were carried out by the lithium acetate procedure (18). All mutations were later confirmed by recovering these plasmids from yeast cells and sequencing the relevant region. Standard *E. coli* (35) and yeast (38) media were used.

**Mating assays.** Both halo assays and quantitative mating assays were performed essentially as described before (43). For halo assays to test **a**-factor secretion, 2-μl aliquots of cultures were spotted onto a lawn of the tester strain XBH8-2C (*MAT*α *sst2*) and incubated for 24 h at 30°C. In all quantitative mating experiments, a fivefold excess of strain GPY60 (*MAT*α *leu2-3,112 ura3-52 his4-579 trp1-289 prb1 gal2 Δpep4*::*URA3*) was used as the mating partner to test Ste6p function. Mating efficiencies were defined as the percentage of diploid cells obtained relative to the total number of *MAT***a** cells available to mate.

**Preparation of Ste6p-specific antibodies.** A 579-bp *Xba*I-*Nar*I fragment from the *STE6* gene, encoding a portion of the protein distal to NBD1, was cloned into the expression vector pATH2 and transformed into *E. coli* MC1061. Expression and isolation of the TrpE-Ste6p fusion protein were carried out essentially as described before (24), and the resulting material was used as an immunogen to generate a rabbit polyclonal antiserum. Alternatively, the fusion protein was extracted from gel slices directly into a buffer containing 0.5 M NaCl and 0.1 M $NaHCO_3$, pH 8.3, prior to being coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) for use as a TrpE-Ste6p fusion protein affinity matrix.

The immune rabbit serum was cut with ammonium sulfate at 50% saturation (16). The precipitate was collected by centrifugation at 10,000 × *g* and dialyzed against a buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4), and 0.02% sodium azide (buffer A). This material was diluted fivefold into buffer A supplemented with 0.1% bovine serum albumin (BSA) (buffer B). It was then loaded onto the TrpE-Ste6p protein affinity matrix preequilibrated in buffer B. The column was washed sequentially with buffer B and buffer B containing 2.0 M urea. The specific anti-TrpE-Ste6p antibodies were eluted with a buffer containing 0.2 M glycine (pH 2.5), 150 mM NaCl, 1 mM EDTA, and 0.1% BSA. The eluate was then immediately neutralized with 1/10 volume of 1 M Tris, pH 8.0. After dialysis into buffer A, the eluate was passed over a nonspecific affinity column made with a total protein extract from the Δ*ste6* strain YDB108. The flowthrough (containing the Ste6p-specific antibodies) was concentrated to the original serum volume with a Centricon 10 microconcentrator (Amicon). It was dialyzed into 1× phosphate-buffered saline (PBS, pH 7.4) and stored at 4°C in the presence of 0.02% sodium azide until use.

**Immunoprecipitations.** Immunoprecipitation of extracellular **a**-factor was carried out essentially as described before (46). Cultures of the yeast strain YDB108 expressing wild-type or mutant *STE6* alleles were grown overnight in selective minimal medium at 30°C. They were then diluted into the same medium and grown at the indicated temperature (30 or 37°C) until a culture density of 0.5 to 1.0 optical density at 600 nm ($OD_{600}$) units/ml was reached. A total of 3.5 $OD_{600}$ units of cells were harvested and resuspended in 0.5 ml of the same medium in a polypropylene tube (Falcon). [$^{35}$S]cysteine (Amersham) was added to a final concentration of 240 μCi/ml, and samples were incubated for 2 h at the indicated temperature (Fig. 3, Table 1). The samples were then incubated for an additional 30 min on ice. The culture tubes were washed twice with distilled water, and **a**-factor was resuspended in *n*-propanol. The samples were then transferred to a 1.5-ml centrifuge tube and lyophilized to dryness under vacuum. Control experiments confirmed that >95% of **a**-factor was recovered by this procedure as previously reported (46). The **a**-factor was resuspended in 50 μl of sodium dodecyl sulfate (SDS) boiling buffer (1% SDS, 1 mM EDTA, 50 mM Tris, pH 7.5) and boiled for 3 min. Then, 0.45 ml of Tween IP buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween 20) containing an excess of **a**-factor-specific polyclonal antiserum (generously provided by Jeff Becker) was added. After a 2-h incubation at room temperature, 75 μl of a 3.6% suspension of protein A-Sepharose was added, and the incubation was continued for an additional 30 min. The beads were washed twice with Tween IP buffer and once with Tween IP buffer supplemented with 2 M urea. The beads were resuspended in 20 μl of SDS sample buffer and boiled for 3 min, and the samples were electro-

phoresed on SDS–12 to 18% polyacrylamide gradient gels. The gels were dried, and **a**-factor-specific counts were quantitated with a PhosphorImager (Molecular Dynamics).

**Immunoblots.** Cultures of yeast strain YDB141 expressing wild-type or mutant *STE6* alleles from the low-copy plasmid pSEYC58 were grown overnight in selective minimal medium at 30°C. Cells were then harvested and resuspended in YPD medium containing α-factor (15 μg/ml), and the culture was grown for an additional 2 h at 30°C. Since Ste6p was prone to aggregation in standard SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (our unpublished results), cells were lysed directly in SDS-PAGE sample buffer containing 6 M urea. Extracts were heated for 5 min at 50°C and immediately loaded onto an SDS–8% PAGE gel. The separated proteins were then transferred to an Immobilon-P membrane (Millipore) with a Trans-Blot apparatus (Bio-Rad). Following a 1-h blocking step in 5% nonfat dry milk dissolved in PBS–0.5% Tween 20, the blot was incubated with affinity-purified Ste6p antibodies, washed, and then incubated with $^{35}$S-protein A (Amersham).

**Indirect immunofluorescence.** Yeast strains expressing either wild-type or mutant forms of Ste6p were cultured as described above for immunoblots. Cycloheximide was added to 0.1 mg/ml for 10 min, and 5 OD units of each strain were then harvested by centrifugation at 630 × *g* in a tabletop centrifuge. Cells were resuspended in 1.0 ml of YPD, immediately dropped into 14 ml of ice-cold methanol, and incubated for 10 min at −20°C. Following centrifugation, cell pellets were resuspended in 0.5 ml of a buffer containing 0.5 mM $MgCl_2$ and 0.1 M potassium phosphate, pH 6.8. The cells were spheroplasted for 30 min at 37°C in the presence of 20 μg of Oxalyticase (Enzogenetics) per ml. The spheroplasts were harvested by a brief centrifugation (30 s at 16,000 × *g*) in a microcentrifuge and washed three times with 1.0 ml of PBS, pH 7.4, containing 0.2% azide (PBSA buffer). The spheroplasts were attached to glass microscope slides and treated with 3% formaldehyde in PBSA buffer for 10 min at room temperature. They were washed three times with PBSA buffer and blocked in 1.0% BSA in PBSA (blocking buffer) for 10 min at room temperature. The samples were incubated for 30 min at 37°C with affinity-purified Ste6p antibodies in blocking buffer. The slides were then washed and subjected to subsequent rounds of blocking and incubation with DTAF (dichlorotiazinylaminofluorescein)-conjugated goat anti-rabbit antibodies (heavy and light chain [H+L]) and DTAF-conjugated donkey anti-goat antibodies (H+L) (Jackson Laboratories). The samples were mounted for visualization in PBSA buffer containing 0.1% phenylenediamine and 90% glycerol and sealed under coverslips.

**Reagents.** α-Factor was synthesized by the University of Alabama at Birmingham Cancer Center peptide synthesis core facility with an Applied Biosystems 430A peptide synthesizer and purified by high-pressure liquid chromatography (HPLC). A 1-mg/ml working stock was prepared in 90% methanol and stored at −20°C until use.

## RESULTS AND DISCUSSION

**Initial characterization of Ste6p mutants.** A number of mutations in or near the LSGGQ motif within the first NBD of CFTR have been shown to cause the disease CF (10, 23, 47). Since this sequence motif is highly conserved among the NBDs of all ABC transporters, it was proposed to mediate a conserved function among these proteins (1). Within a 39-amino-acid region that includes the LSGGQ motifs in the first NBDs of CFTR and Ste6p, the amino acid sequence homology is significant, 46% identity (69% similarity), between Ste6p and CFTR (Fig. 1B). Since distinct mutations within this region of CFTR cause large differences in the severity of CF, we hypothesized that these CF-related mutations introduced into the corresponding positions of Ste6p would result in a broad range of **a**-factor transport and mating defects. It was previously shown that the introduction of one severe CF-related mutation in the LSGGQ motif of either NBD of Ste6p (G509D or G1349D) results in severe defects in both **a**-factor transport and mating (6). To further correlate the level of transported **a**-factor with the efficiency of mating, we introduced nine CF-related missense mutations in or near the LSGGQ motif of the first NBD of Ste6p.

To characterize the effects of each of these mutations, low-copy (*CEN*) and high-copy (2μm) plasmid vectors carrying each of these mutant constructs were transformed into yeast strain YDB108, which harbors a deletion-disruption of the chromosomal *STE6* gene. Transformants expressing either wild-type or mutant forms of Ste6p were initially assayed for the ability to transport **a**-factor in an **a**-factor halo assay (43).

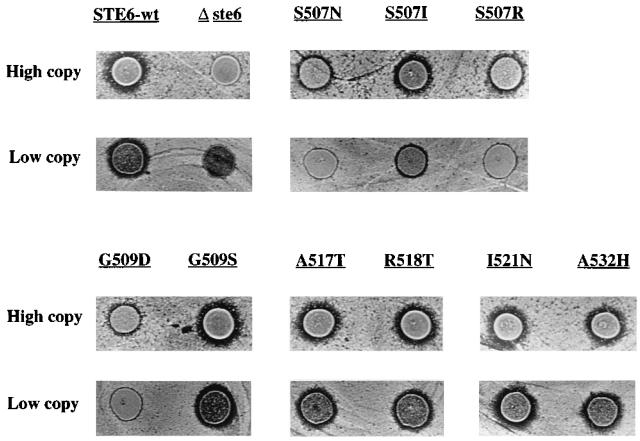


FIG. 2. Halo assays to detect **a**-factor secretion by yeast strains expressing wild-type (wt) or mutant forms of Ste6p. Aliquots of cultures expressing the indicated forms of Ste6p from low-copy or high-copy plasmids were spotted onto a lawn of the **a**-factor tester strain XBH8-2C (*MAT*α *sst2*). The plates were then incubated for 24 h at 30°C.

In this assay, a *MAT***a** strain is spotted onto a lawn of a *MAT*α *sst2* tester strain and incubated at 30°C. Since growth of the tester strain is inhibited by **a**-factor, a zone of growth inhibition (a halo) appears in the lawn around cells that actively secrete **a**-factor. The size of the halo is proportional to the amount of pheromone transported.

A large halo was seen surrounding cells expressing wild-type Ste6p from either a low-copy or high-copy plasmid (Fig. 2). As expected, little or no growth inhibition was seen around the Δ*ste6* strain, which synthesized **a**-factor but was unable to transport it out of the cell. Strains expressing Ste6ps containing the G509S, A517T, R518T, I521N, or A532H mutation generated halos similar in size to that of the strain expressing wild-type Ste6p even when the mutant transporters were expressed from a low-copy plasmid. In contrast, strains carrying low-copy plasmids expressing Ste6ps with the LSGGQ mutation S507N, S507I, S507R, or G509D produced halos that were clearly reduced in size, indicating significant reductions in **a**-factor transport. Even when Ste6p was overproduced from a high-copy plasmid, strains expressing Ste6ps carrying the S507N, S507R, or G509D allele made noticeably smaller halos than the wild-type control. In contrast, the halo made by the strain expressing the Ste6p mutant S507I from a high-copy plasmid was similar to that of the wild-type strain, indicating that this mutation caused a less severe **a**-factor transport defect.

**Quantitation of a-factor transport by Ste6p mutants.** We next quantitated the level of **a**-factor transported by yeast cells expressing each of the Ste6p mutants from a low-copy plasmid at 30°C. Each strain was labeled with [$^{35}$S]cysteine, and the labeled extracellular **a**-factor was recovered by immunoprecipitation with an **a**-factor-specific antiserum (Fig. 3). These results were quantitated and expressed as the amount of **a**-factor transported by each mutant form of Ste6p relative to the amount of **a**-factor transported by wild-type Ste6p (Table 1). The relative level of **a**-factor transported by strains expressing the mutant forms of Ste6p ranged from 7 to 97% at 30°C. The most severe defects in **a**-factor transport were associated with mutations located directly within the LSGGQ motif. The S507N, S507R, and G509D mutations reduced **a**-factor transport 12- to 15-fold. The less severe S507I mutation resulted in a fivefold drop in transported **a**-factor. The G509S mutation caused only a twofold drop in **a**-factor transport, while the

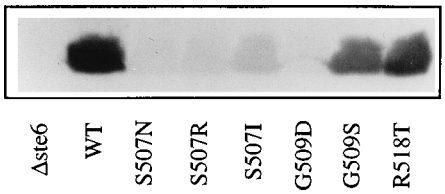


FIG. 3. Immunoprecipitation of extracellular **a**-factor transported by wild-type (WT) and mutant Ste6ps expressed from low-copy plasmids at 30°C.

A517T, A532H, R518T, and I521N mutations, located just outside the LSGGQ motif, supported essentially wild-type levels of **a**-factor transport at 30°C.

To further characterize the effects of these mutations on Ste6p function and to reveal any further defects in **a**-factor transport that were not readily apparent at 30°C, we next examined **a**-factor transport by these strains at 37°C. However, we found that the overall level of **a**-factor transported by wild-type Ste6p was reduced four- to fivefold at this temperature, which made it difficult to quantitate the small amount of **a**-factor transported by yeast cells expressing several of the Ste6p mutants. We therefore examined the feasibility of increasing the total level of transported **a**-factor by introducing a high-copy plasmid bearing the *MFa1* gene into each strain. Under these conditions, the amount of **a**-factor transported from yeast cells expressing wild-type Ste6p increased more than sixfold at 30°C, demonstrating that both the synthesis and transport of mature **a**-factor were increased. These results confirmed that Ste6p was not rate limiting for **a**-factor transport in these strains, consistent with previous reports that **a**-factor transport increases four- to eightfold when yeast cells are transformed with high-copy plasmids encoding either the *MFa1* or *MFa2* gene (19, 28). Similarly, the introduction of the high-copy plasmid bearing *MFa1* into the strain expressing wild-type Ste6p increased **a**-factor transport at 37°C to a level that was twofold above the level transported by yeast cells lacking the *MFa1* plasmid at 30°C.

TABLE 1. **a**-Factor transport of strains carrying *STE6* mutations[a]

| *STE6* allele | Relative **a**-factor transport (% of control) ± SD | | | Transport ratio, 30°C/37°C |
|---|---|---|---|---|
| | Low-copy *MFa1*, 30°C | High-copy *MFa1* | | |
| | | 30°C | 37°C | |
| Wild type (control) | 100 | 100 | 100 | 1.0 |
| S507N | 6.6 ± 3.1 | 8.5 ± 1.9 | 1.1 ± 0.7 | 7.7 |
| S507R | 8.3 ± 6.7 | 10 ± 5.2 | 7.3 ± 2.2 | 1.4 |
| S507I | 20 ± 9.2 | 18 ± 12 | 10 ± 0.2 | 1.8 |
| G509D | 7.3 ± 3.1 | 6.5 ± 1.5 | 8.4 ± 2.3 | 0.8 |
| G509S | 52 ± 8.1 | 61 ± 23 | 56 ± 2.7 | 1.1 |
| A517T | 97 ± 16 | ND[b] | ND | |
| R518T | 91 ± 5.7 | 95 ± 1.8 | 23 ± 7.5 | 4.1 |
| I521N | 82 ± 15 | ND | ND | |
| A532H | 92 ± 16 | ND | ND | |

[a] Relative **a**-factor transport is the mean level of extracellular **a**-factor transported by each mutant Ste6p expressed as a percentage of the extracellular **a**-factor transported by wild-type Ste6p under the same conditions. In each case, the trace amount of extracellular **a**-factor detected in the Δ*ste6* culture was subtracted as background. Each value represents the data from at least four independent determinations.

[b] ND, not determined.

We next asked whether the overproduction of **a**-factor affected the relative levels of **a**-factor transported by yeast cells expressing wild-type and mutant forms of Ste6p at 30°C. In all cases, our results indicated that the relative levels of **a**-factor transport remained the same (Table 1). This established that the overproduction of **a**-factor at 30°C resulted in an increase in the total amount of transported **a**-factor without altering the relative differences in **a**-factor transport among the various constructs. The strains overproducing **a**-factor were then used to analyze the effect of the Ste6p mutants on **a**-factor transport at 37°C. Our results indicate that the relative amount of **a**-factor transported by most of the mutant forms of Ste6p examined (S507R, S507I, G509D, and G509S) closely paralleled the relative level of pheromone transported by each of these mutant proteins at 30°C. However, yeast cells expressing the R518T and S507N Ste6ps exhibited relative levels of extracellular **a**-factor that were four- and eightfold less, respectively, when assayed at 37°C. This suggests that these two mutations caused temperature-sensitive defects in **a**-factor transport.

**Quantitative assays of mating phenotypes associated with decreased a-factor transport.** The relative mating efficiencies of strains expressing either the wild-type or mutant forms of Ste6p were next examined in a quantitative mating assay (43). In this assay, yeast strains of opposite mating type are allowed to mate for 5 h under optimal conditions and then plated on a selective medium that supports the growth of only **a**/α diploid cells. With this protocol, millions of diploid cells can be formed in a single assay by using a *MAT***a** strain expressing wild-type Ste6p from a low-copy plasmid. In an identical assay with the isogenic Δ*ste6* strain as a mating partner, no diploid cells are formed.

Quantitative mating assays were first carried out at 30°C with yeast cells expressing either wild-type or mutant forms of Ste6p from a low-copy plasmid. The LSGGQ mutations caused a broad range of mating defects at 30°C (Table 2). Each of three Ste6p mutations at the serine residue within the LSGGQ motif (S507N, S507R, and S507I) resulted in mating defects at 30°C. The S507N mutation caused a 90-fold decrease in mating relative to the wild-type control. The S507R mutation reduced mating ninefold below that with the wild-type Ste6p control, while the S507I mutation reduced the mating efficiency only twofold. The introduction of a mutation at the second glycine residue of the LSGGQ motif, G509D, caused a severe (56-fold) decrease in mating at 30°C relative to the wild-type control. This result is similar to the mating defect previously reported for this mutation in Ste6p (6). In contrast to the G509D mutation, however, a G509S mutation resulted in a wild-type level of mating at 30°C. Strains expressing forms of Ste6p carrying mutations at more distal residues (A517T, R518T, I521N, and A532H) were all found to mate with wild-type efficiencies at 30°C. Taken together, these data indicate that the most severe mating defects were associated with mutations directly within the LSGGQ motif.

We next examined the mating efficiency of strains expressing wild-type or mutant forms of Ste6p from a low-copy plasmid at 37°C. It was previously shown that mating of *S. cerevisiae* increases as the temperature is raised to 34°C but is significantly reduced at 36°C (37). Consistent with this finding, the mating efficiency of cells expressing wild-type Ste6p decreased from 70% at 30°C to 3.5% at 37°C (a 20-fold drop). When strains expressing the mutant forms of Ste6p were examined at 37°C, we found that several mutations caused much more severe mating defects (relative to the strain expressing wild-type Ste6p) than was observed at 30°C (Table 2). For example, the mating efficiencies of strains expressing the S507R and S507N Ste6ps at 37°C were 10,000-fold less than that of the isogenic

TABLE 2. Mating of strains carrying *STE6* mutations[a]

| *STE6* allele | Relative mating efficiency (% of control ± SD) | | Ratio, 30°C/37°C |
|---|---|---|---|
| | 30°C | 37°C | |
| Wild type (control) | 100 | 100 | 1.0 |
| S507N | 1.1 ± 1.2 | 0.0092 ± 0.012 | 120 |
| S507R | 11 ± 9 | 0.0073 ± 0.012 | 1,507 |
| S507I | 50 ± 16 | 0.97 ± 0.95 | 52 |
| G509D | 1.8 ± 1.5 | 0.025 ± 0.027 | 72 |
| G509S | 91 ± 26 | 50 ± 32 | 1.8 |
| A517T | 70 ± 44 | 41 ± 24 | 1.7 |
| R518T | 85 ± 32 | 9.4 ± 2.5 | 9 |
| I521N | 104 ± 34 | 38 ± 23 | 2.7 |
| A532H | 115 ± 21 | 47 ± 44 | 2.4 |

[a] The relative mating efficiency is the mean mating efficiency of each mutant Ste6p expressed as a percentage of the mating efficiency mediated by wild-type Ste6p at the same temperature. The Δ*ste6* control did not allow diploid formation at either temperature. Each value represents the data from five to seven independent mating assays.

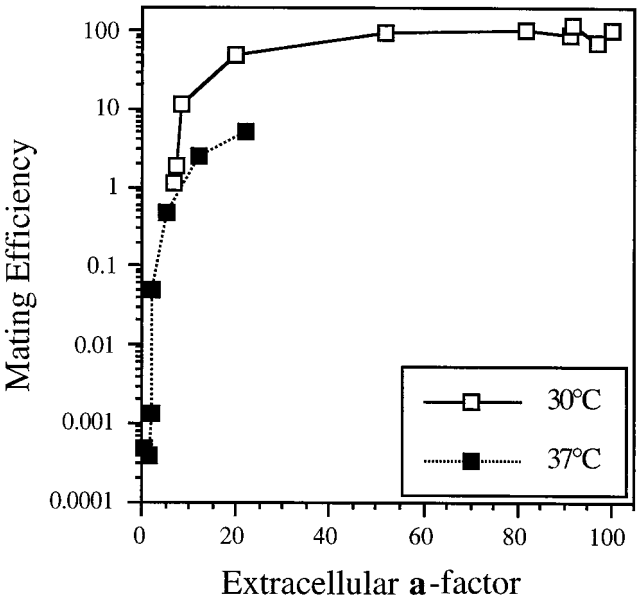


FIG. 4. Correlation between the relative level of extracellular **a**-factor and the relative efficiency of yeast mating at 30 and 37°C. Values for both mating efficiency and extracellular **a**-factor are percentages of those observed with cells expressing wild-type Ste6p at 30°C.

wild-type strain assayed under the same conditions. Likewise, the strain expressing the S507I form of Ste6p mated at a frequency that was 100-fold lower than that of the wild-type control when assayed at 37°C, while mating of the strain expressing the G509D form of Ste6p was 4,000-fold lower than that of the wild-type control. In contrast, the strain expressing the G509S form of Ste6p as well as several mutations distal to the LSGGQ motif (A517T, I521N, and A532H) exhibited only a two- to threefold decrease in mating at 37°C. Only one distal mutation (R518T) was found to cause a significant (11-fold) reduction in mating at 37°C, which is consistent with the temperature-sensitive **a**-factor transport associated with this mutation.

**Correlation between the level of transported a-factor and mating.** The results of the above experiments with strains expressing wild-type and mutant forms of Ste6p from a low-copy plasmid were next used to express the relative mating efficiencies as a function of extracellular **a**-factor levels at both 30 and 37°C (Fig. 4). Under conditions of limiting extracellular **a**-factor at 30°C, the mating efficiency undergoes a large decrease in response to a small drop in the concentration of extracellular **a**-factor. For example, a threefold decrease in extracellular **a**-factor, from 20% (S507I) to 7% (S507N), correlated with a 50-fold drop in mating efficiency. Only 20% of the **a**-factor transport mediated by wild-type Ste6p was required for maximal mating and thus represented a saturating level of pheromone when presented in this physiological context at 30°C.

The correlation between the mating response observed as a function of transported **a**-factor at 37°C revealed certain differences compared with the results obtained at 30°C. First, the absolute amount of **a**-factor required for maximal mating approached a plateau near the same saturating level of pheromone observed at 30°C, indicating that the level of transported **a**-factor required to promote a maximal level of mating does not change significantly at the higher temperature. However, the total amount of **a**-factor transported at 37°C by the strain expressing wild-type Ste6p from a low-copy plasmid was only 22% of that obtained at 30°C, indicating that total **a**-factor transport is reduced at 37°C. In addition, the absolute level of mating promoted by this amount of transported **a**-factor is 10-fold lower than that observed at 30°C, indicating that one or more distal steps of the mating pathway are much less responsive to pheromone stimulation at the elevated temperature. Under conditions of limiting extracellular **a**-factor at 37°C, large decreases in mating efficiency were again observed in response to a small drop in **a**-factor transport. Because the total amount of **a**-factor transported by Ste6p is reduced at 37°C, the combined data for the two temperatures shown in Fig. 4 provide a correlation between the efficiency of yeast mating as a function of transported **a**-factor over a 400-fold range of **a**-factor concentrations.

**Stability and subcellular localization of LSGGQ mutants.** Mutations in the *STE6* gene could decrease **a**-factor transport by reducing the steady-state level of Ste6p, by altering its proper localization to cell surface, or by directly altering its transport properties. In order to distinguish between these possibilities, we first asked whether the low **a**-factor transport associated with the most severe Ste6p LSGGQ mutations could be correlated with a reduced steady-state level of Ste6p. Extracts were prepared from yeast cells grown at 30°C that expressed wild-type, S507N, S507R, S507I, or G509D Ste6p from a low-copy plasmid, and the amount of Ste6p present in each strain was determined by immunoblot analysis. Mean values from several experiments similar to the one shown in Fig. 5 were determined by PhosphorImager analysis. We found that the abundance of each of these mutant proteins was similar to the abundance of wild-type Ste6p, indicating that the **a**-factor transport defects caused by these LSGGQ mutations are not attributable to a decrease in the steady-state level of Ste6p.

We next asked whether the **a**-factor transport defects associated with the LSGGQ mutations were attributable to a defect in the transit of Ste6p to its normal location at (or near) the cell surface. Unfortunately, Ste6p does not undergo detectable glycosylation during its movement through the secretory pathway (27), so progressive carbohydrate addition and/or processing could not be used to determine the subcellular location of mutant forms of Ste6p. Previous studies have shown that the half-life of wild-type Ste6p is normally quite short (15 to 30

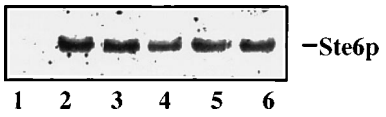


FIG. 5. Steady-state levels of wild-type and mutant Ste6ps. Lanes: 1, Δ*ste6*; 2, wild type; 3, S507N; 4, S507R; 5, S507I; 6, G509D. Yeast cells expressing the indicated forms of Ste6p from low-copy plasmids were grown overnight at 30°C in minimal medium. Cells were then harvested, resuspended in YPD medium supplemented with α-factor (15 μg/ml), and cultured for an additional 2 h at 30°C. Cells were then harvested, and extracts were subjected to immunoblot analysis with affinity-purified Ste6p antibodies.

min). During its brief life span, it is transported through the secretory pathway to the cell surface and then rapidly endocytosed to the vacuole, where it is degraded (5, 25). Because of the rapid nature of Ste6p's endocytosis upon reaching the cell surface, most Ste6p is normally located in transport vesicles at any given time rather than at the cell surface. However, both the half-life and the surface accumulation of Ste6p are increased in strains defective for endocytosis (5, 25). To determine whether the LSGGQ mutations resulted in either intracellular retention or mislocalization of Ste6p, we compared the subcellular localizations of the wild-type protein and the two most severe mutants by indirect immunofluorescence. To do this, high-copy plasmids carrying the wild-type, S507N, or G509D allele of the *STE6* gene were introduced into a Δ*ste6* strain that also harbored the *act1-1* mutation. The *act1-1* mutation is a temperature-sensitive allele of the single actin gene in *S. cerevisiae*. This mutation was previously shown to severely reduce the rate of endocytosis of surface proteins (26). The strains were exposed to α-factor at the semipermissive temperature of 30°C, and affinity-purified Ste6p-specific antibodies were used to determine the subcellular localization of Ste6p in these strains by indirect immunofluorescence. We observed accumulation of each of the mutant Ste6ps at (or near) the cell surface in the *act1-1* mutant strains in a manner that was indistinguishable from the accumulation of the wild-type protein (Fig. 6A). This suggests that these mutant proteins are properly transported to the cell surface, where they accumulate as a consequence of the endocytosis defect.

When *MAT***a** yeast strains are exposed to α-factor, the synthesis of **a**-factor is increased and the transport of secretory vesicles is directed to a projection of the cell, which causes the cell to acquire a pear-shaped morphology (45). In these cells, Ste6p is concentrated predominantly near the surface of the projection. (It should be noted that such projections are not observed in the *act1-1* strain shown in Fig. 6A because proper assembly of the actin cytoskeleton is required to achieve this elongated shape.) To further examine the localization of the Ste6p mutants, cells expressing wild-type and mutant forms of Ste6p were exposed to α-factor to induce the formation of these projections, and these cells were then examined by indirect immunofluorescence with Ste6p-specific antibodies (Fig. 6B). Strains expressing either the wild-type or mutant forms of Ste6p showed the characteristic localization of Ste6p to the projection, again suggesting that the subcellular localization of each of these mutant forms of Ste6p is normal. Since the LSGGQ mutations were found not to affect either the stability or the localization of Ste6p, we conclude that the lack of **a**-factor transport is most likely attributable to a direct defect in some aspect of Ste6p's transporter function.

The efficient completion of yeast mating depends upon the coordination of a series of complex physiological processes such as fusion site selection, agglutination, growth arrest, and gene activation and expression (30, 45). Each of these processes is activated in the presence of the appropriate effector pheromone. Several previous studies have investigated the sensitivities of these processes to exogenously added **a**-factor and α-factor (4, 8, 19–21, 31, 32). In general, it was found that exogenously added pheromone is unable to induce an efficient mating response. In this study, we used *STE6* mutations to titrate the presentation of extracellular **a**-factor in its proper physiological context and found a strong correlation between the level of **a**-factor transported by Ste6p and the efficiency of yeast mating. It has been proposed that the level of **a**- and α-factor secreted by *MAT***a** and MATα yeast cells, respectively, acts as the primary determinant of each haploid cell's identification as an appropriate mating partner by the target cell. As each cell secretes its respective pheromone, a concentration gradient decreasing away from the secreting cell is established. These gradients were proposed to provide the positional cues necessary for mating partner and fusion site selection (19–21, 31). Our results indicate that *MAT***a** yeast cells must export at least 20% of the level of **a**-factor transported by wild-type Ste6p (when expressed from a low-copy plasmid) for maximal recognition by *MAT*α yeast cells in a quantitative mating assay. Below this saturating level of **a**-factor, the efficiency of mating decreased drastically when **a**-factor transport was further reduced. This confirms that *MAT*α cells can efficiently discriminate between small differences in the level of **a**-factor presented by *MAT***a** cells and is consistent with the model in which a concentration gradient of secreted **a**-factor plays an important role in mating partner selection. This mechanism would provide an efficient means by which *MAT*α cells filter out the stimulus from more distant *MAT***a** cells (or those secreting lower levels of **a**-factor) during the mating process.

Recently, a new class of *STE6* mutations that caused only modest **a**-factor transport defects but conferred a relatively severe defect in cell fusion were reported (12). From these results, it was suggested that Ste6p may carry out other functions necessary for mating other than **a**-factor transport (12). In our study, we found a direct correlation between the level of **a**-factor transport by Ste6p and the efficiency of yeast mating. The most straightforward interpretation of these results is that the LSGGQ motif of Ste6p is involved primarily in **a**-factor transport. However, we cannot exclude the possibility that other regions of Ste6p may be involved in additional functions related to the overall mating process.

While the LSGGQ motif is highly conserved throughout the ABC transporter superfamily, its functional significance is unknown. Some mutations within the LSGGQ motif of CFTR cause retention (and subsequent degradation) of newly synthesized CFTR in the endoplasmic reticulum of mammalian cells (9, 15). The possibility that these mutations simply result in a folding defect recognized by the quality control apparatus of the endoplasmic reticulum has raised doubts about the possible role that this sequence element may play in the function of ABC transporters. However, studies with another ABC transporter, the histidine permease of *Salmonella typhimurium*, have suggested that the LSGGQ motif may act as a signal transducer between the hydrophobic domain and the NBD of that transporter (1). Our results also suggest that this region may be directly involved in Ste6p's transporter function, a role that would be consistent with the strong evolutionary conservation of this element within the ABC transporter superfamily. Further studies are required to elucidate the specific role of the LSGGQ motif in ABC transporter function.

## ACKNOWLEDGMENTS

This work was supported by grants from the Cystic Fibrosis Foundation (R464) and NIH (RO1 DK50832). We acknowledge the sup-

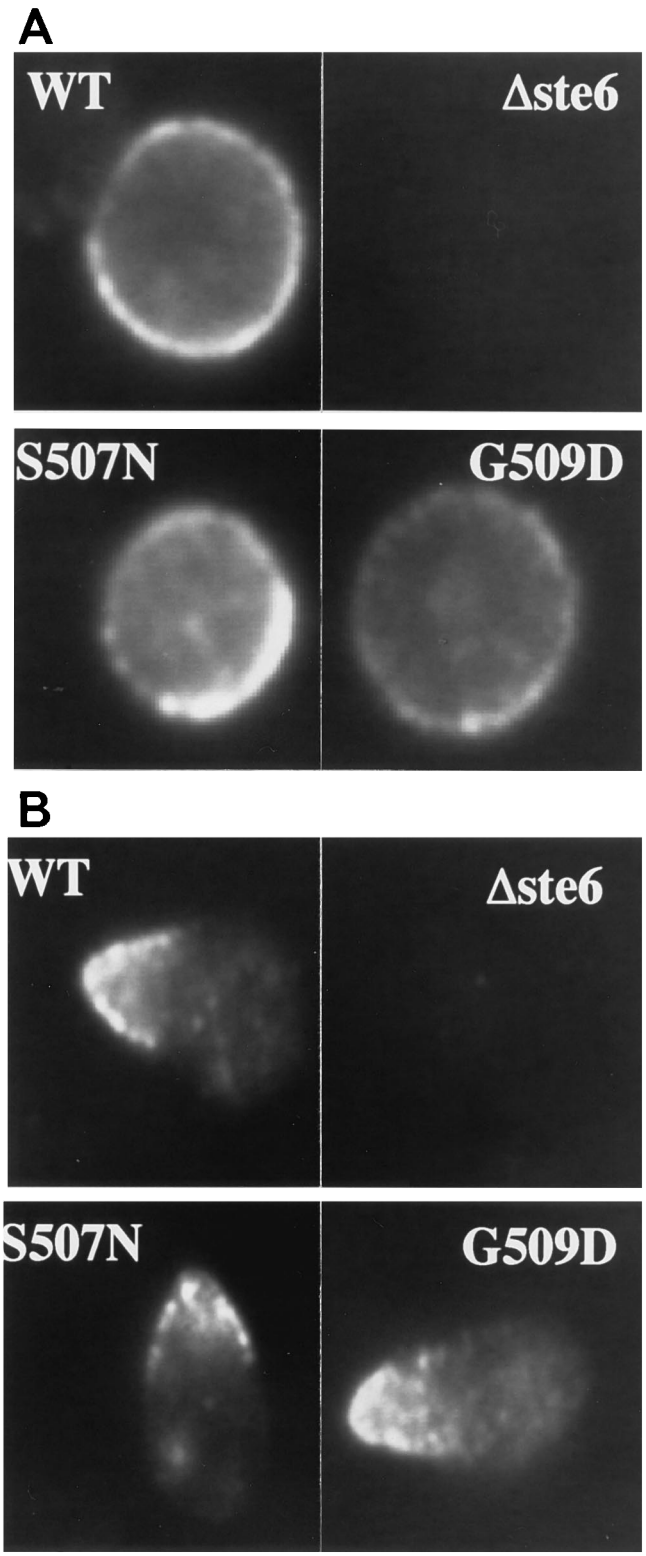


FIG. 6. Subcellular localization of wild-type (WT) and mutant Ste6ps by indirect immunofluorescence. (A) Localization of Ste6p in the *act1-1* mutant strain YDB199. (B) Localization of Ste6p to cell surface projections in the wild-type strain YDB141 following α-factor treatment. Yeast cells expressing the indicated forms of Ste6p from high-copy plasmids were grown overnight at 25°C (A) or 30°C (B) in minimal medium. Cells were then harvested, resuspended in YPD medium supplemented with α-factor (15 μg/ml), and incubated for an additional 2 h at 30°C. The cells were then fixed and processed for immunofluorescence as described in Materials and Methods.

port of the University of Alabama at Birmingham Center for AIDS Research (P30 AI27767) for access to the Genetics Computer Group package of sequence analysis programs and the University of Alabama at Birmingham Comprehensive Cancer Center (P30 CA13148) for use of the peptide synthesis core facility.

We thank Jeff Becker and Guy Caldwell for providing the *MFa1* clone and an **a**-factor-specific antiserum and Susan Michaelis for providing the pBR322-*STE6* clone and an Ste6p-specific antiserum used in preliminary experiments. We also thank Jeff Becker, Guy Caldwell, Vytas Bankaitis, Susan Michaelis, Scott Emr, and Jeff Stack for helpful discussions and Nicole Owens for excellent technical assistance.

## REFERENCES

1. **Ames, G. F.-L., C. S. Mimura, S. R. Holbrook, and V. Shyamala.** 1992. Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. Adv. Enzymol. **65:**1–47.
2. **Ames, G. F.-L., C. S. Mimura, and V. Shyamala.** 1990. Bacterial periplasmic permeases belong to a family of transport proteins operating from *E. coli* to human: traffic ATPases. FEMS Microbiol. Rev. **75:**429–447.
3. **Anderegg, R. J., R. Betz, S. A. Carr, J. W. Crabb, and W. Duntze.** 1988. Structure of *Saccharomyces cerevisiae* mating hormone **a**-factor: identification of *S*-farnesyl cysteine as a structural component. J. Biol. Chem. **263:** 18236–18240.
4. **Baffi, R. A., P. Shenbagamurthi, K. Terrance, J. M. Becker, F. Naider, and P. N. Lipke.** 1984. Different structure-function relationships for α-factor-induced morphogenesis and agglutination in *Saccharomyces cerevisiae*. J. Bacteriol. **158:**1152–1156.
5. **Berkower, C., D. Loayza, and S. Michaelis.** 1994. Metabolic instability and constitutive endocytosis of Ste6p, the **a**-factor transporter of *Saccharomyces cerevisiae*. Mol. Biol. Cell **5:**1185–1198.
6. **Berkower, C., and S. Michaelis.** 1991. Mutational analysis of the yeast **a**-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily. EMBO J. **10:**3777–3785.
7. **Brake, A., C. Brenner, R. Najarian, P. Laybourn, and J. Merriweather.** 1985. Structures of genes encoding precursors of the yeast peptide mating pheromone **a**-factor, p. 103–108. *In* M.-J. Gething (ed.), Current communications in molecular biology: protein transport and secretion. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
8. **Caldwell, G. A., S.-H. Wang, C.-B. Xue, Y. Jiang, H.-F. Lu, F. Naider, and J. M. Becker.** 1994. Molecular determinants of bioactivity of the *Saccharomyces cerevisiae* lipopeptide mating pheromone. J. Biol. Chem. **269:**19817–19826.
9. **Cheng, S. H., R. J. Gregory, J. Marshall, S. Paul, D. W. Souza, G. A. White, C. R. O'Riordan, and A. E. Smith.** 1990. Defective intracellular transport and processing is the molecular basis of most cystic fibrosis. Cell **63:**827–834.
10. **Cutting, G. R., L. M. Kasch, B. J. Rosenstein, J. Zielenski, L.-C. Tsui, S. E. Antonarakis, and H. H. Kazazian.** 1990. A cluster of cystic fibrosis mutations in the first nucleotide binding fold of the cystic fibrosis conductance regulator protein. Nature (London) **346:**366–369.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
12. **Elia, L., and L. Marsh.** 1995. Genes required for cell fusion during mating, p. 21. *In* Cold Spring Harbor Yeast Cell Biology Meeting Abstracts. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Emr, S. D., A. Vassarotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas.** 1986. The amino terminus of the yeast F1-ATPase β subunit precursor functions as a mitochondrial import signal. J. Cell Biol. **102:**523–533.
14. **Geitz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
15. **Gregory, R. J., D. P. Rich, S. H. Cheng, D. W. Souza, S. Paul, P. Manavalan, M. P. Anderson, M. J. Welsh, and A. E. Smith.** 1991. Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. Mol. Cell. Biol. **11:**3886–3893.
16. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
17. **Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gill, and M. P. Gallagher.** 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomembr. **22:**571–592.
18. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
19. **Jackson, C. L., and L. H. Hartwell.** 1990. Courtship in *S. cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. Cell **63:**1039–1051.
20. **Jackson, C. L., and L. H. Hartwell.** 1990. Courtship in *Saccharomyces cerevisiae*: an early cell-cell interaction during mating. Mol. Cell. Biol. **10:**2202–2213.
21. **Jackson, C. L., J. B. Konopka, and L. H. Hartwell.** 1991. *S. cerevisiae* α pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. Cell **67:**389–402.

22. **Julius, D., R. Schekman, and J. Thorner.** 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell **36:**309–318.
23. **Kerem, B.-S., J. Zielenski, D. Markiewicz, D. Bozon, E. Gazit, J. Yahav, D. Kennedy, J. R. Riordan, F. S. Collins, J. M. Rommens, and L.-C. Tsui.** 1990. Identification of mutations in regions corresponding to the two putative nucleotide (ATP) binding folds of the cystic fibrosis gene. Proc. Natl. Acad. Sci. USA **87:**8447–8451.
24. **Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff.** 1991. High expression vectors with multiple cloning sites for construction of *trpE* fusion proteins. Methods Enzymol. **194:**477–490.
25. **Kolling, R., and C. P. Hollenberg.** 1994. The ABC transporter Ste6 accumulates in the plasma membrane in a ubiquinated form in endocytosis mutants. EMBO J. **13:**3261–3271.
26. **Kubler, E., and H. Riezman.** 1993. Actin and fimbrin are required for the internalization step of endocytosis in yeast. EMBO J. **12:**2855–2862.
27. **Kuchler, K., H. G. Dohlman, and J. Thorner.** 1993. The **a**-factor transporter (*STE6* gene product) and cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. **120:**1203–1215.
28. **Kuchler, K., R. Sterne, and J. Thorner.** 1989. *Saccharomyces cerevisiae STE6* gene product: a novel pathway for protein export in eukaryotic cells. EMBO J. **8:**3973–3984.
29. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
30. **Kurjan, J.** 1992. Pheromone response in yeast. Annu. Rev. Biochem. **61:** 1097–1129.
31. **Marcus, S., G. A. Caldwell, D. Miller, C.-B. Xue, F. Naider, and J. M. Becker.** 1991. Significance of C-terminal cysteine modifications to the biological activity of the *Saccharomyces cerevisiae* **a**-factor mating pheromone. Mol. Cell. Biol. **11:**3603–3612.
32. **Marsh, L.** 1992. Substitutions in the hydrophobic core of the α-factor receptor of *Saccharomyces cerevisiae* permit response to *Saccharomyces kluyveri* α-factor and to antagonist. Mol. Cell. Biol. **12:**3959–3966.
33. **McGrath, J. P., and A. Varshavsky.** 1989. The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature (London) **340:**400–404.
34. **Michaelis, S., and I. Herskowitz.** 1988. The **a**-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. Mol. Cell. Biol. **8:**1309–1318.
35. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Mimura, C. S., S. R. Holbrook, and G. F.-L. Ames.** 1991. Structural model of the nucleotide-binding conserved component of periplasmic permeases. Proc. Natl. Acad. Sci. USA **88:**84–88.
37. **Reid, B. J., and L. H. Hartwell.** 1977. Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. J. Cell Biol. **75:**355–365.
38. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–209.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:**430–434.
42. **Schafer, W. R., and J. Rine.** 1992. Protein prenylation: genes, enzymes, targets, and functions. Annu. Rev. Genet. **26:**209–237.
43. **Sprague, G.** 1991. Assay of yeast mating function. Methods Enzymol. **194:** 77–93.
44. **Sprague, G., and I. Herskowitz.** 1981. Control of yeast cell type by the mating type locus: identification and control of expression of the **a**-specific gene *BAR1*. J. Mol. Biol. **153:**305–321.
45. **Sprague, G. F., and J. W. Thorner.** 1992. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*, p. 657–744. *In* E. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Sterne, R. E.** 1989. A novel pathway for peptide hormone biogenesis: processing and secretion of the mating pheromone **a**-factor by *Saccharomyces cerevisiae*. Ph.D. thesis. University of California, Berkeley.
47. **Strong, T. V., L. S. Smit, S. V. Turpin, J. L. Cole, C. T. Hon, D. Markiewicz, T. L. Petty, M. W. Craig, E. C. Rosenow III, L.-C. Tsui, M. C. Iannuzzi, M. R. Knowles, and F. S. Collins.** 1991. Cystic fibrosis gene mutation in two sisters with mild disease and normal sweat levels. N. Engl. J. Med. **325:**1630–1634.
48. **Tsui, L.-C.** 1992. Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the Cystic Fibrosis Genetic Analysis Consortium. Hum. Mutat. **1:**197–203.
49. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **8:**945–951.
50. **Wilson, K. L., and I. Herskowitz.** 1984. Negative regulation of *STE6* gene expression by the α2 product of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**2420–2427.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.